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ABSTRACT

The aim of this proposal is to investigate the contribution of NF-κβ signaling within macrophages in defining the macrophage's role in normal and neoplastic mammary development. The proposed experiments use multiple murine lines and novel assay systems. We have been developing novel murine models with which to address this issue. We are continuing to use our established IκBα and transplantation technology to investigate the effects of constitutive NF-κB activity within macrophages on mammary development and believe that elevated NF-κB may result in increased macrophage recruitment. To more effectively monitor NF-κB activity within specific cell types we have undergone a collaborative effort and generated a novel Luciferase/GFP reporter transgenic. The inducible model system that we proposed to use in our studies requires three transgenic components; an inducible dominant inhibitor, an inducible constitutive activator and a macrophage specific expressor of the reverse transactivator protein. We have generated and confirmed the functionality of the first two transgenics and are characterizing the third. We are making good progress towards the goal of the study.

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INTRODUCTION

Morphogenesis of the mammary gland is a highly complex process which when misregulated can result in tumorigenesis. It involves the interactions of multiple cell types in a highly regulated manner with complex signal transduction pathways coordinating the physiological processes. Interactions between epithelial and mesenchymal cells are known to be important. However, recent studies are beginning to highlight the importance of other cell types, such as macrophages. One of the signaling molecules that appears to be critical in regulating the dynamic changes during normal and neoplastic development is the nuclear factor-kappa B (NF-κB) family of transcription factors. NF-κB can regulate many genes that are expressed by macrophages that are important for proliferation and apoptosis of cells, as well as remodeling and angiogenesis on tissue. This proposal seeks to investigate the contribution of NF-κB signaling within macrophages in defining the macrophage's role in normal and neoplastic mammary development. The proposed experiments use multiple murine lines and novel assay systems. These studies will provide information regarding the signaling pathways involved in normal mammary development. They will also provide insights into the importance of NF-κB signaling in macrophages for tumor development and progression and will have the potential for identification of novel therapeutic strategies.

BODY

Task 1. To investigate the effects of constitutive NF-kappaB activity within macrophages on mammary development (Months 1-24):

a. Perform fetal liver cell transplantation using IkappaB-alpha null and control donors at postnatal day 19 to determine effects on virgin postnatal mammary development of constitutive NF-kappaB activity in reconstituted hematopoietic cells (Months 1-24). [100 mice]
b. Perform fetal liver cell transplantation using IkappaB-alpha null and control donors into recipients at 6 weeks. Allow hematopoetic cell reconstitution for a further 6 weeks. Mate recipient mice and investigate effects on development during pregnancy of constitutive NF-kappaB activity in hematopoietic cells (Months 1-24). [100 mice]

Our lab and others have generated IκBa deficient mice (Chen et al., 2000; Chen et al., 2000b; Beg et al., 1995; Klement et al., 1996). In these animals the major inhibitor of NF-κB is absent resulting in constitutive activity. Neonatal lethality precludes the study of adult mammary gland morphogenesis in these animals. However, fetal liver transplantation and reconstitution of hematopoetic cell lineages enables the effects of constitutive NF-κB activation within these lineages to be investigated in an adult animal (Chen et al., 2000; Everhart et al., 2005).
We have started a program of fetal liver transplantations and very preliminary data suggests that constitutive NF-κB in IκBα -/- reconstituted virgin animals may result in decreased ductal proliferation with heterozygote knockout animals displaying an intermediate phenotype. This result would be the reverse of what we had predicted and we have limited numbers at this stage so we are continuing our investigations. Our attempts to obtain pregnant reconstituted animals have proved thus far unsuccessful. We are attempting to decrease the dosage of radiation to try to find the balance between a practical level of reconstitution while retaining the fertility of the reconstituted animal. We have investigated mammary development during pregnancy in wildtype (+/+) versus heterozygote (+/-) IκBα knockout animals. On examination of H&E stained 16.5dpc sections, we were intrigued to observe increased ductal development in heterozygote animals relative to controls. We had previously believed there to be no significant phenotype in heterozygote knockout animals but given this latest observation we decided to look more closely at the heterozygotes during pregnancy relative to control wildtype

animals. As the involvement of macrophages in both normal development and tumorigenesis is the major focus of this grant, we were curious to determine whether constitutive NF-κB activation in the whole animals without transplantation had an effect on tissue macrophages. In order to investigate the presence of macrophages we have used an F4/80 antibody to stain tissue sections (Figure 1). We believe that the preliminary results suggest that increased numbers of macrophages are found in tissue with constitutive NF-κB activity and are currently exploring which downstream signals may be responsible for this phenotype.

In our original proposal we were planning to measure the *in vivo* effects of modulating NF-κB activity in macrophages using our HLL reporter transgenics that express luciferase under an NF-κB responsive promoter such that luciferase assay of crude protein extracts can be used to quantify NF-κB activity. While the HLL reporter mice have provided valuable information regarding NF-κB activation *in vivo*, three issues have led us to construct a second generation of NF-κB reporter transgenic mice. First, although we have attempted to identify NF-κB activation at a cellular level, we have been unable to reproducibly identify luciferase protein or mRNA in individual cells despite repeated efforts with immunohistochemistry and *in situ* hybridization. Second, we would like to be able to sort individual cells that have activated NF-κB. Third, the proximal HIV-LTR is an NF-κB dependent promoter that by all indications has good fidelity as a read-out for NF-κB activation in the models we have used to date; however, it is still a formal possibility that other transcription factors, including Sp1, could influence transcription of this promoter. Therefore, we decided that it would be valuable to have an additional line of reporter mice with another NF-κB dependent promoter to confirm and validate findings using the HLL mice. These issues have led us to collaborate with another group here at Vanderbilt to make a new line of transgenic reporter mice that express a green fluorescent protein (GFP)/luciferase fusion protein under the control of a synthetic NF-κB dependent promoter. Since GFP can be detected in individual cells, these reporter mice allow cell specific determination of NF-κB directed transcription, a crucial factor in understanding NF-κB dependent downstream signaling. The luciferase moiety of the fusion protein also enables luciferase assay from protein extracts and *in vivo* imaging as described above for the HLL reporter transgenics.

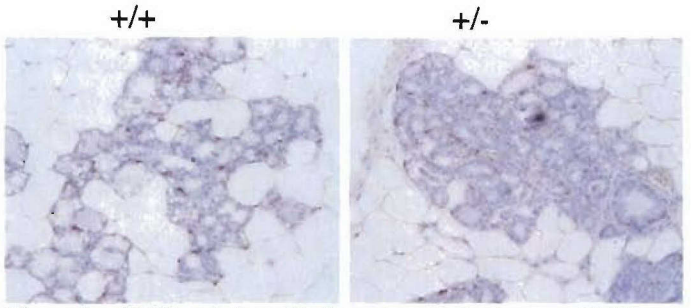

Figure 1 – F4/80 staining of IκB-α +/- and +/+ tissue at 16.5dpc shows increased macrophage presence in +/- animals relative to controls.

The new transgenic NF-κB reporter mice contain tandem copies of a 36 base enhancer from the 5' HIV-LTR that includes both NF-κB binding sites. The proximal 169 base pairs of these promoter constructs, including the TATA box, are from the herpes simplex virus thymidine kinase promoter. We constructed plasmids for expression of enhanced GFP (EGFP)/luciferase fusion protein under the control of 8 replicates of the NF-κB enhancer sequence. The CMV promoter present in pEGFP/Luc (Clontech, Palo Alto, CA) has been replaced with our synthetic NF-κB dependent promoter. The 8x construct was microinjected by the Vanderbilt Transgenic/ES Cell Shared Resource to generate transgenic lines (C57Bl6/DBA background) named NGL [for NF-κB-GFP/Luciferase]. Our initial characterization has shown that the NGL reporter transgenics can be used in the same manner as the HLL, giving comparable results. The new NGL transgenics are better than the HLL transgenics as they can also enable visualization of specific cell types in which NF-κB is activated by fluorescence microscopy or immunohistochemistry of tissue sections. Activation of NF-κB results in expression of GFP protein that can be detected by fluorescence microscopy or by immunohistochemical analysis of paraffin embedded sections using an anti-GFP antibody. We have tested the efficacy of the new NGL mice by crossing them with transgenics that elevate NF-κB activity in the mammary epithelium. The mice produced

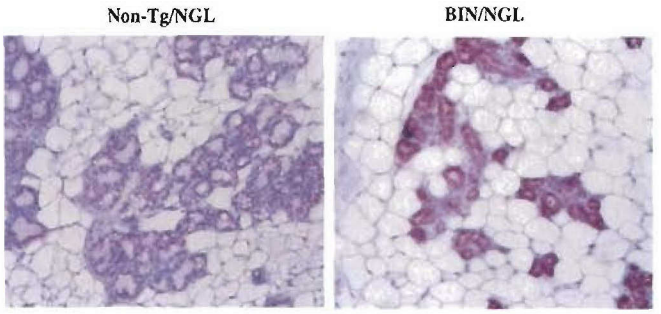

Figure 2 - Immunohistochemistry for GFP expression in single NGL transgenic and double transgenics. Shown are examples of mammary tissue at 16.5dpc.

from these matings allow us to visualize where the NF-κB is active (Figure 2). The representative examples shown illustrate how it is possible to see both the histological structure of the tissue and to localize and get an indication of NF-κB activity. At 16.5dpc, in an NGL transgenic ie. an animal that is effectively wild-type as far as mammary development is concerned but carries the NF-κB reporter, there is diffuse NF-κB activity throughout the epithelial tissue that is proliferating and filling the mammary fat pad. In double transgenics we are able to visualize both the level and localization of NF-κB activity and determine the effect of the transgene that is modulating NF-κB activity. In the double transgenic animals the level of NF-κB activity is seen to be higher but is confined to a more dense area. In summary, we have been focusing our efforts on the development of these new reporter transgenics that are already providing the kind of data that was hitherto not possible to collect. We are continuing our investigations into the effects resulting from the fetal liver transplantation technology but have decided that increased information will be obtained by introducing the NGL reporter transgene into the IκBα animals used in these studies, therefore, we have been breeding the reporter transgene into the knockout lines.

Task 2. Assess effects on mammary development of induced and inhibited NF-kappaB activity using novel inducible transgenics (Months 1-36).

a. Investigate postnatal development in doxycycline-induced macrophage-restricted constitutive activator double transgenic mice (IKMRP) and controls (Months 1-36). [300 mice]
b. Investigate postnatal development doxycycline-induced macrophage-restricted dominant inhibitor double transgenic mice in (DNMRP) and controls (Months 1-36). [300 mice]

In collaboration with another group, we are developing conditional transgenic modular mouse models, based on the tetracycline inducible system, to over-express IκB-DN (dominant inhibitor) or cIKK2 (constitutive activator) in response to treatment with doxycycline in drinking water. The components of this system include transgenics expressing the reverse tetracycline transactivator (rtTA) in the appropriate cell type, and transgenics in which the tetracycline operator (tet-O)$_7$ and a minimal CMV promoter drive expression of either the dominant inhibitor (IκB-DN), or a constitutively active IKK2 mutant. Key DNA components for the system were obtained from Dr Jay Tichelaar (Perl et al., 2002). In the presence of doxycycline, the rtTA binds to tet-O and induces downstream gene expression within 24 hours. In these transgenics, the IκB-DN construct that we have has mutations of the critical phosphorylation targets (serine residues) that are normally phosphorylated in response to signaling resulting in degradation of the inhibitor (Chen et al., 1999). This mutated form of inhibitor is not degraded in response to phosphorylation signals and therefore functions to block NF-κB signaling. To facilitate detection of induced transgene products we have attached a FLAG tag to the cIKK2 and a Myc-His tag to the dominant inhibitor. The transgenics expressing the inhibitor are named DN. The transgenics expressing the activator are named IKK. In order to modulate expression in macrophages , we have been collaborating with the group of Dr. John Christman. We have designed transgenics with macrophage restricted expression of the reverse tetracycline transactivator (rtTA)(Perl et al., 2002). Expression is restricted to macrophages using a

promoter based on the mannose receptor promoter (MRP)(Eichbaum et al., 1997) and including 3 repeats of a PU.1 responsive element (DeKoter et al., 2000). The transactivator transgenics are named MRP. With the assistance of the Vanderbilt Transgenic/ES Cell Shared Resource we have generated 4 independent lines each of the DN, IKK and MRP transgenics. In order to confirm inducible transgene expression in response to doxycycline treatment we have crossed the DN lines with an existing, well-characterized lung-specific CC-10rtTA transgenic (Perl et al., 2002). Animals were treated with doxycycline in drinking water (1mg/ml) for 3 days prior to collection of tissue samples. We have demonstrated doxycycline induction of DN expression as measured by western analysis in lung tissue (Figure 3A). Results show no uninduced expression in the absence of doxycycline in the drinking water and no detectable non tissue-specific expression, therefore the system is not leaky.

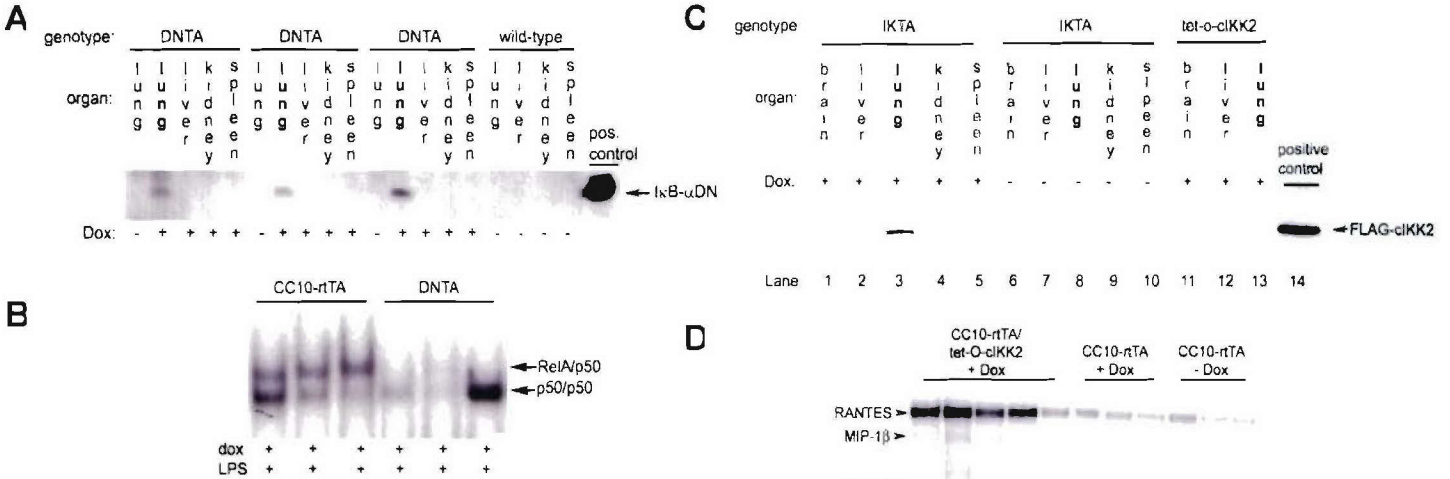

Figure 3A – western analysis using tissues from doxycycline induced and uninduced DN:CC-10-rtTA transgenic mice show non-leaky tissue specific induction of DN transgene. The CC-10 promoter directs expression to lung epithelium.
B – electrophoretic mobility shift assay showing NF-κB binding activity in control single CC10-rtTA transgenics produced by LPS treatment and inhibition of NF-κB binding activity in the presence of the induced DN transgene in double transgenics.
C – western analysis in tissues from doxycycline induced and uninduced IKK:CC-10-rtTA transgenic mice show non-leaky

The induced DN transgene is also able to inhibit NF-κB activation in the lung in response to LPS treatment that would normally be expected to increase NF-κB activity and hence binding in a gel mobility shift assay (Figure 3B). We have generated 4 lines of IKK transgenics. In equivalent crosses with rtTA transgenics to those decribed above, we have confirmed lung specific expression of the constitutive activator upon doxycycline administration and the ability of the transgenic protein to enhance NF-κB activation in the lung (Figure 3C and D). Thus, we have generated transgenic lines in which the expression of the NF-κB dominant

inhibitor or constitutive activator transgenes are inducible in the presence of the transactivator protein and administration of doxycycline in the drinking water. The DN and IKK transgene products are functional and able to modulate NF-κB activity *in vivo*.

We have generated 4 independent lines of MRP transgenics. We have been able to detect the rtTA mRNA using RT-PCR in alveolar macrophages harvested from bronchiolar lavage fluid (Figure 4).

We have also detected message in bone marrow - derived macrophage cultures (data not shown). We have generated double transgenic animals carrying a combination of the DN and MRP transgenes (DNMP) or the IKK and MRP transgenes (IKMP). A small number of double transgenics and controls have been treated with doxycycline to induce transgene expression. Our initial attempts to detect expression of the transgenes by western analysis of macrophages harvested from bronchiolar lavage fluid have been unsuccessful. We are currently culturing bone marrow macrophages from double transgenic animals and appropriate controls in order to obtain a larger population of cells to use for western analysis.

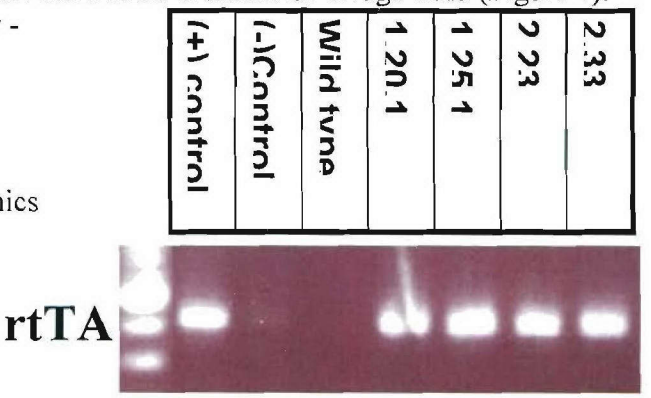

Figure 4: Expression of rtTA as detected by RTPCR

While our modular inducible transgenic system has not yet been employed in macrophages, we have confirmed efficacy of the DN and IKK component transgenics in the lung epithelium and therefore, expect the system will be effective once we have the macrophage rtTA component transgenics.

Task 3. To determine whether manipulation of NF-kappaB activity within macrophage populations effects tumorigenesis (Months 25-36).

This task is dependent on establishing the model in task 2 and is not scheduled to commence until month 25.

KEY RESEARCH ACCOMPLISHMENTS

1) Initial results suggest that fetal liver reconstitution using IκBα deficient cells may result in decreased ductal development
2) Early data suggests that in IκBα +/- mice, constitutive NF-kappaB activity may increase macrophage presence during pregnancy
3) In collaboration with the group of Dr. Timothy Blackwell, we have generated a novel GFP/luciferase transgenic reporter of *in vivo* NF-kappaB activity that we can use to obtain information concerning NF-kappaB activity at the resolution of single cells on tissue sections.
4) In collaboration with the group of Dr. Timothy Blackwell, we have generated transgenics in which the dominant inhibitor of NF-kappaB is under the control of an inducible promoter and confirmed that they are functional.
5) In collaboration with the group of Dr. Timothy Blackwell, we have generated transgenics in which a constitutive activator of NF-kappaB is under the control of an inducible promoter and confirmed that they are functional.

6) In collaboration with the group of Dr. John Christman, we have generated several lines of transgenics designed to target expression of the reverse transactivator protein (rtTA) to macrophages and are commencing their characterization.

REPORTABLE OUTCOMES

In a collaborative effort with the laboratory of Dr. Timothy Blackwell we have developed three novel transgenics; an in vivo reporter of NF-kappaB activity, an inducible dominant inhibitor of NF-kappaB activity and an inducible constitutive activator of NF-kappaB activity.

CONCLUSIONS

We have generated and confirmed efficacy of three novel transgenic lines that represent powerful tools for the investigation of the *in vivo* roles of NF-kappaB. A fourth type of transgenic, the final component necessary to use our inducible model system in macrophages, is being characterized. Very early results suggest that NF-kappaB activity within macrophages is important for mammary ductal development. In conclusion we are making good progress towards our stated goals.

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APPENDICES

None